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Identification of H-2K^b-Restricted T-Cell Epitopes Within the Nucleocapsid Protein of Hantaan Virus and Establishment of Cytotoxic T-Cell Clones

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Although neutralizing antibodies against Hantaan virus (HTV) can protect hosts from viral infection, T-cell responses to HTV are also important in host defense against HTV. However, much less is known about cytotoxic T lymphocyte (CTL) responses to HTV. To identify CTL epitopes in the HTV nucleocapsid protein (NP), we selected 7 H-2K^b-motif-fitting peptides. Of these peptides, 3 peptides (NP3, NP4, and NP7) were recognized by CTL responses derived from HTV-immunized mouse splenocytes. NP3 and NP4 peptides were also recognized by HTV-immunized splenocytes after secondary in vitro stimulation with the relevant peptide, but NP7 could not be recognized after in vitro stimulation. These results agree well with peptide immunization studies showing that peptide-specific CTL responses could be induced with NP3 and NP4 but not with NP7 peptide. Furthermore, CTL activity assay using targets, prepared to express the antigen (NP) endogenously, demonstrated that NP3 and NP4 peptides could be presented endogenously. CTL elicited with NP4 peptide retained some cross-reactivity and was difficult to long-term culture. However, NP3-elicited CTL was very specific for NP3 peptide and was stable enough to be cloned. Among many CTL lines elicited with HTV or HTV NP peptides, 6 NP3-specific CTL clones were established and have been maintained more than 2 years. All 6 CTL clones were characterized to be CD3+, CD4–, CD8+, CD25+, CD62L–, and NK1.1–, and to use TCR Vβ6. This preferential usage of TCR Vβ6 indicates that TCR Vβ6 regions are important for recognition of the HTV NP3 epitope (NP221–228, SVIGFLAL) on H-2K^b molecule. Our data demonstrate the definition of mouse CTL epitopes in HTV and the generation of HTV-specific mouse CTL clones. ***J. Med. Virol. 60: 189–199, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: Hantaan virus; nucleocapsid protein; epitope; CTL cloning; TCR Vβ usage

INTRODUCTION

Hantaan virus (HTV) is a member of the genus *Hantavirus* of the family Bunyaviridae and a causative agent of hemorrhagic fever with renal syndrome (HFRS) [Lee et al., 1981]. These viruses have single-stranded, negative sense RNAs as 3 genome segments that encode the polymerase: 2 envelope glycoproteins (G1, G2) and the nucleocapsid protein (NP), respectively [Tamura et al., 1989]. HTV is maintained in nature through asymptomatic infections of *Apodemus agrarius,* which is known to be a natural primary rodent reservoir [Kariwa et al., 1995]. Because the transmission of HTV to humans is via aerosols of contaminated excreta such as urine, feces, or saliva from infected mice, much interest has focused on the way in which the virus is maintained and spread among rodent hosts [Chu et al., 1995]. Although suitable animal models that mimic the HFRS are presently unavailable, various animal experiments have been carried out to address the availability of an animal model of the HFRS. Consequently, systemic infection leading to lethal outcome was observed in newborn mice [Kim and McKee, 1985; McKee et al., 1985; Nakamura et al., 1985a], nude mice [Nakamura et al., 1985b], and SCID

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mice [Yoshimatsu et al., 1997], which have immature or defective immune systems.

It is well known that neutralizing antibodies, especially to G1 and G2, play major roles for protection against HTV infection [Zhang et al., 1988; Schmaljohn et al., 1990; Yoshimatsu et al., 1993]. Those circulating antibodies are thought to prevent the primary infection of the virus in host animals but do not contribute to the clearance of the virus that has already multiplied in the host cells. Kariwa et al. [1995] found that HTV could replicate or survive for a certain period in adult mice in spite of the presence of the specific antibody. Although a high level of neutralizing antibody was present in suckling mice inoculated with HTV, the virus persisted in the animal for several weeks [Nakamura et al., 1985b]. Furthermore, it was reported that neutralizing monoclonal antibody-escape mutants of HTV were generated in the presence of antibodies to G1 and G2 proteins [Kikuchi et al., 1998]. Therefore, it seems that efficient protection against HTV infection could not be provided by neutralizing antibodies alone and thereby needs the cellular immune system.

NP of HTV, which is antigenically and genetically more conserved than the envelope proteins, has been applied mainly as a diagnostic antigen. Therefore, the report [Schmaljon et al., 1990] showing that baculovirus recombinants expressing only NP could protect animals from HTV challenge is very interesting. It suggests that the NP elicited a nonneutralizing, perhaps T-cell-mediated, protective immune response. Human CD4+ and CD8+ cytotoxic T lymphocyte (CTL) epitopes on NP have been identified in Sin Nombre virus (SNV) [Ennis et al., 1997] and recently in HTV [Van Epps et al., 1999]. Other viral NPs also have been reported to be recognized by CTL responses in viral infection such as lymphocytic choriomeningitis virus (LCMV), mouse hepatitis virus (MHV), or influenza virus [Deckhut et al., 1993; Bergmann and Stohlman, 1996].

Although it is believed that CTL activities are important for resistance of mice to HTV infection, mouse CTL epitopes or CD8+ CTL clones have not been reported. Hitherto, protection against or clearance of HTV infection by CTLs was described only with bulk cultures of splenocytes from mice immunized with HTV [Nakamura et al., 1985a; Asada et al., 1987, 1988]. Thus precise CTL epitopes or CTL clones could not be determined. In this study, we have identified CTL epitopes in HTV NP to gain a better understanding of the recognition of HTV by cellular immune responses. CD8+ CTL clones specific for HTV NP epitope were also established through in vitro restimulation of splenocytes from C57BL/6 mice immunized with HTV or NP221–228 epitope peptide followed by limit-dilution and long-term culture. The epitope-based approach offers potential advantages over the more conventional approach using whole CTLs, such as clarifying the role of epitopes and specific CTL clones in HTV infection. To our knowledge, this is the first report about mouse CTL epitopes in HTV and CD8+ CTL clones specific for NP of HTV.

MATERIALS AND METHODS

Mice and Cells

C57BL/6 mice and ICR mice were purchased from Charles River Laboratories (Wilmington, MA) and 6–10-week-old female mice were used for studies except for suckling mice. EL-4, P815, YAC-1, and Vero E6 cells were obtained from American Type Culture Collection (ATCC; Monassas, VA). All cells, except for Vero E6 cells, which were cultured in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), were cultured at 37°C in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS, L-glutamine (2 mM), streptomycin (50 μg/ml), penicillin (50 U/ml), HEPES buffer (20 mM), and 1% nonessential amino acids.

Virus

HTV (strain 76-118), obtained from ATCC, was passaged 2 times in Vero E6 cell culture and used for further passages. HTV was inoculated intracerebrally (i.c.) into suckling ICR mice within 24 hours after birth. Three further passages were performed in suckling mice by using 10% suspensions of brain tissues prepared from moribund animals. The resultant working stock, consisting of a 10% suspension of virus-infected brain tissue, was frozen at −70°C until use. Titers of focus-forming units (ffu) of the stock virus were determined by the focus reduction neutralizing assay method, as described previously [Yoo et al., 1993]. The experiments, which involved infection of mice with live HTV, were performed according to standard biosafety level 3 conditions.

Phenotyping of CTL Clones by Flow Cytometry

T lymphocytes were stained in Phosphate-buffered saline (PBS) containing 0.02% sodium azide with antibodies purchased from Pharmingen (San Diego, CA). Nonspecific binding to Fc receptors was blocked with anti-CD16/CD32 (FcγIII/II receptor). The various lymphocyte markers were characterized by using phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies to CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD25 (PC61), CD62L (MEL-14), Pan-NK (DX5), and NK1.1 (PK136). Cells (5×10^5/sample in 100 μl) were preincubated with anti-CD16/CD32 and further incubated with anti-CD markers for 30 minutes in ice-cold water and washed with cold PBS. Analysis was performed on FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CELLQUEST (Becton Dickinson). Machine compensation and channel sensitivity were standardized using Calibrite Beads (Becton Dickinson). The lymphocyte population was gated by forward scatter (fsc)/side scatter (ssc) analysis to exclude dead cells.

Synthetic Peptides

Peptides were synthesized by a solid-phase method using the Fmoc-based protocol on an automated synthesizer (model 430A, Applied Biosystems, Foster City,

CA). The crude products were purified on a reverse-phase preparative high performance liquid chromatographic (HPLC) column (Vydac, Hesperia, CA). Homogeneity of the final products was assessed by analytical HPLC. Peptides were characterized by amino acid analysis system (Hewlett Packard, Wilmington, DE) and electrospray mass spectrometry on a Platform II from VG (Manchester, UK). Pure peptide fractions were lyophilized and dissolved at 0.5–2 mg/ml in PBS.

Immunization and In Vitro CTL Stimulation

To prepare the peptide immunogen, a lipid film was made by mixing phosphatidylethanolamine palmitoyl oleoyl (POPE) and cholesterol hemisuccinate at a 7:3 molar ratio followed by drying on nitrogen gas [Zhou et al., 1994]. Then peptide solution was added at a 1:20 molar ratio of peptide to lipid and gently resuspended with the lipid film. From this suspension, small unilamella vesicles (SUV) were prepared by sonication for 3 hours. To enhance encapsulation efficiency and stability, the vesicles were frozen and thawed repeatedly and stored in liquid nitrogen until used. C57BL/6 (H-2^b) mice were immunized intraperitoneally (i.p.) with HTV (0.1 ml containing 10^5 ffu) once, or with peptide immunogens (100 μg/0.1 ml/dose) 2 or 3 times at 7-day intervals. Two or 3 mice were used per immunization. Splenocytes were pooled for primary CTL activities. Spleens were removed 7 days after the last injection and depleted of erythrocytes by NH_4Cl (0.83%) hypotonic solution before rinsing. Splenocytes were cultured for 5–7 days in RPMI 1640 containing 10% FBS (HyClone, Logan, UT), 5×10^{-5} M 2-ME (2-mercaptoethanol), 100 μg/ml streptomycin, 100 U/ml penicillin 2 mM L-glutamine, and nonessential amino acids (Gibco-BRL, Grand Island, NY). Bulk-cultured CTLs showing peptide-specific cytotoxicity were restimulated in culture medium containing 5 ng/ml mIL-2 (Calbiochem, La Jolla, CA), 10 μg/ml peptide, and syngeneic splenocytes irradiated at 2,500 rad as antigen presenting cells (APC). If necessary, this restimulation was performed every week and cytotoxic activity was assessed by CTL assay.

Separation of CD8+ T Cells

CD8+ T lymphocytes were isolated as described in the manual provided by Miltenyi Biotech (Sunnyvale, CA). Briefly, enriched CTL bulk cultures, showing peptide-specific cytotoxicity for about 2 months, were incubated with microbeads directly conjugated with anti-CD8 mAb (Miltenyi Biotech) and positively selected by miniMACS system (Miltenyi Biotech).

Establishment and Maintenance of CD8+ CTL Clones

Clones were obtained from CD8+ CTLs by a limiting-dilution technique as described previously [Rogers et al., 1991]. Graded numbers of CTLs (0.1–50 cells) were cultured in wells of 96-well plates containing irradiated (2,500 rad) syngeneic spleen cells (5×10^5 cells), 10 μg/ml peptide, and 5 ng/ml mIL-2 in 0.2 ml of culture medium. Cultures were refed every 3 days by replacing 50% of the volume in each well with fresh culture medium. After 10–18 days, wells were scored visually for growing cells. Proliferating cell clusters were chosen for further expansion only when the frequency of positive wells at a given cell dilution was less than 20%. Wells containing more than one distinct cell cluster were not selected for further expansion to ensure that the expanded cultures were the progeny of individual clonal precursors. Monoclones were expanded by transferring to individual wells of 24-well plates and thereafter 6-well plates in the presence of peptide-pulsed APC and mIL-2 as above. This cloning procedure was repeated 3 times, until CD8+ CTL clones showed stable proliferation and continuous cytotoxicity specific for the peptide. CTL clones were maintained by restimulation every 2 weeks.

Preparation of Target Cells and Cytotoxicity Assay

The cytotoxic activity of CTL culture and monoclones was assessed in a standard 4-hour cytotoxic assay with ^{51}Cr-labeled target cells as previously described [Feltkamp et al., 1994; Oh et al., 1998]. Target cells used in this study were peptide-pulsed EL-4 cells, HTV-inoculated peritoneal exudate cells (PEC; actually macrophage), HTV NP-transfected EL-4 cells, and control cells (P815, YAC-1). Syngeneic macrophages were prepared and inoculated with HTV according to the procedure of others with some modifications [Asada et al., 1988]. First, macrophages were harvested by washing the peritoneal cavity of C57BL/6 mice pretreated with 4.0% thioglycollate 3–4 days before. After overnight incubation in Teflon-coated 100 mm Petri dishes at 37°C, the adherent cells, of which the major population of cells are macrophages, were inoculated with HTV at 0.5 ffu/cell and incubated at 37°C for 2 hours. The virus-inoculated macrophages were cultured in fresh medium for 5 days after rinsing with PBS. HTV infection into macrophages was confirmed by immunofluorescence assay (IFA) [Kurata et al., 1983]. EL-4 cells (H-2^b) were transfected with pcDNA3.1 vector (Invitrogen, San Diego, CA) into which HTV nucleocapsid open reading frame had been introduced using DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N,-trimethyl ammonium methylsulfate) (Boehringer Mannheim, Mannheim, Germany). Transfected cells were screened in RPMI 1640 medium supplemented with 0.5 mg/ml G418 (Gibco-BRL). Expression of transfected NP was confirmed by IFA and Western blot [Chu et al., 1995]. Target cells were washed and resuspended in culture media. Target cells (10^4/well) were incubated with effector CTLs at different ratios in U-bottomed 96-well plates. Supernatants were collected 4 hours after incubation at 37°C, and radioactivity from each well was evaluated by a gamma-counter, CobraII from Packard Instrument (Meriden, CT). Specific CTL lysis was represented as: percentage specific lysis = 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). All of the CTL assays

TABLE I. Recognition of NP Peptides by CTL Responses of C57BL/6 Mice

Peptide	Amino acid No.[a]	Sequence[a]	H-2K^b binding score[b]	Specific lysis (%) at E/T ratio[c] 5:1	1:1
NP1	94–101	SMLSYGNV	2.6	10.0	2.1
NP2	121–128	SIIVYLTS	2.6	6.6	3.1
NP3	221–228	SVIGFLAL	11.0	32.0	16.9
NP4	328–335	LGAFFSIL	14.5	39.1	21.6
NP5	376–383	LGQRIIVL	5.2	13.0	4.8
NP6	402–409	MDPELRTL	3.6	7.7	3.2
NP7	422–429	SNQEPLKL	5.7	22.3	12.5
		HTV-infected PEC[d]		42.4	29.3
		HTV NP-transfected EL-4[d]		35.2	26.5

[a]Corresponding to HTV (76-118) NP.
[b]Binding score in the H-2K^b restriction, expressed as arbitrary units, was calculated by a computer program of BIMAS, NIH [Parker et al., 1994].
[c]As targets, EL-4 cells were pulsed with each peptide at a concentration of 10 μg/ml. Splenocytes from C57BL/6 mice infected with HTV (10^5 ffu/head) were used as effector cells. Two mice were used in each experiment and spleens from them were pooled for CTL assay. Specific lysis was calculated by subtracting the lysis of control cells (EL-4 or PEC) from the lysis of target cells.
[d]As endogenous target cells, HTV-infected PEC and NP-transfected EL-4 were used as described in Materials and Methods. Data represent the average of triplicate assays. The results shown are from a representative of 3 separate experiments.

were performed in triplicate and repeated at least twice. Spontaneous release was less than 20% of the total.

TCR Vβ Typing of CTLs

TCR Vβ usage of CTLs was analyzed by flow cytometry. Commercially available monoclonal antibodies recognizing each type of TCR Vβ were used to stain TCR of CTLs. TCR Vβ phenotype was analyzed by FACSCalibur and CELLQUEST (Becton Dickinson). TCR Vβ-specific antibodies, which directly conjugated with PE or FITC, were obtained from Pharmingen [Vβ2 (B20.6), Vβ3 (KJ25), Vβ5 (MR9-4), Vβ6 (RR4-7), Vβ8.3 (1B3.3), Vβ9 (MR10-2), Vβ10 (B21.5), Vβ12 (MP11-1), Vβ13 (MR12-3), Vβ14 (14-2)], and others [Vβ4 (KT4), Vβ7 (TR310), Vβ8.1, 8.2 (KJ16), Vβ11 (KT11)] were purchased from Serotec (Oxford, UK).

RESULTS

Identification of the H-2K^b-Restricted Peptides on the NP Recognized by HTV-Immunized Mice

To identify contiguous 8-amino-acid peptide stretches that could potentially bind to the H-2K^b molecule, we evaluated the sequence of NP of HTV strain 76-118 using an algorithm, provided by Bioinformatics & Molecular Analysis Section (BIMAS) of the National Institutes of Health (NIH) [Parker et al., 1994]. The binding score represents the estimate or half-life of dissociation of a molecule containing that sequence. Thus a higher score means a stronger binder to major histocompatibility complex (MHC). For example, the binding score of chicken ovalbumin peptide (OVA 257–264, SIINFEKL), known as a good binder to H-2K^b, was 17.4; OVA 257–264 peptide is also well known to induce CTL responses efficiently. Screening of the amino acid sequence of the HTV NP yielded 7 peptides showing binding scores ≥2.6 (Table I). We synthesized these peptides and tested their antigenicity in spite of the lower binding score of some peptides. This will increase the probability of epitope selection. To examine whether these peptides elicit the CTL responses in a natural HTV infection in mice, we tested whether peptides could be recognized by primary antiviral CTL responses. HTV-infected PEC and HTV NP-transfected EL-4 cells were also used as target cells, which would express antigens endogenously. As shown in Table I, the results reveal that NP3, NP4, and NP7 peptides sensitized target cells for lysis by anti-HTV CTLs. Those primary anti-HTV CTLs also lysed endogenous target cells. This result implies that these 3 NP peptides play a role in the primary antiviral responses in mice after infection with HTV.

We next examined whether these NP peptides could stimulate anti-HTV CTL population and increase peptide specificity of CTLs. To investigate this possibility, we measured secondary CTL activity after in vitro stimulation of HTV-immunized splenocytes. Splenocytes from HTV-infected C57BL/6 mice were restimulated in vitro for 2 weeks with each peptide, NP3, NP4, and NP7. Syngeneic splenocytes, prepulsed with relevant peptide and irradiated, were used as APC for the last stimulation. A 2-week in vitro restimulation was performed to remove nonspecific T lymphocytes, which usually do not proliferate after 2 weeks of in vitro culture. As expected, restimulation with higher scoring peptides (NP3 and NP4) resulted in strong CTL responses (Fig. 1A,B). However, significant CTL activity could not be found after restimulation with NP7 (Fig. 1C) or without peptide (Fig. 1D). Other peptides than these 3 NP peptides also were tested, but CTL activities could not be detected. These results suggest that primary anti-HTV CTLs require in vitro restimulation with peptide to maintain cytotoxic activity for more than 2 weeks, and peptides NP3 and NP4 are able to provide this restimulation. Of the 7 H-2K^b-motif peptides that we predicted, 3 peptides (NP3, NP4, and

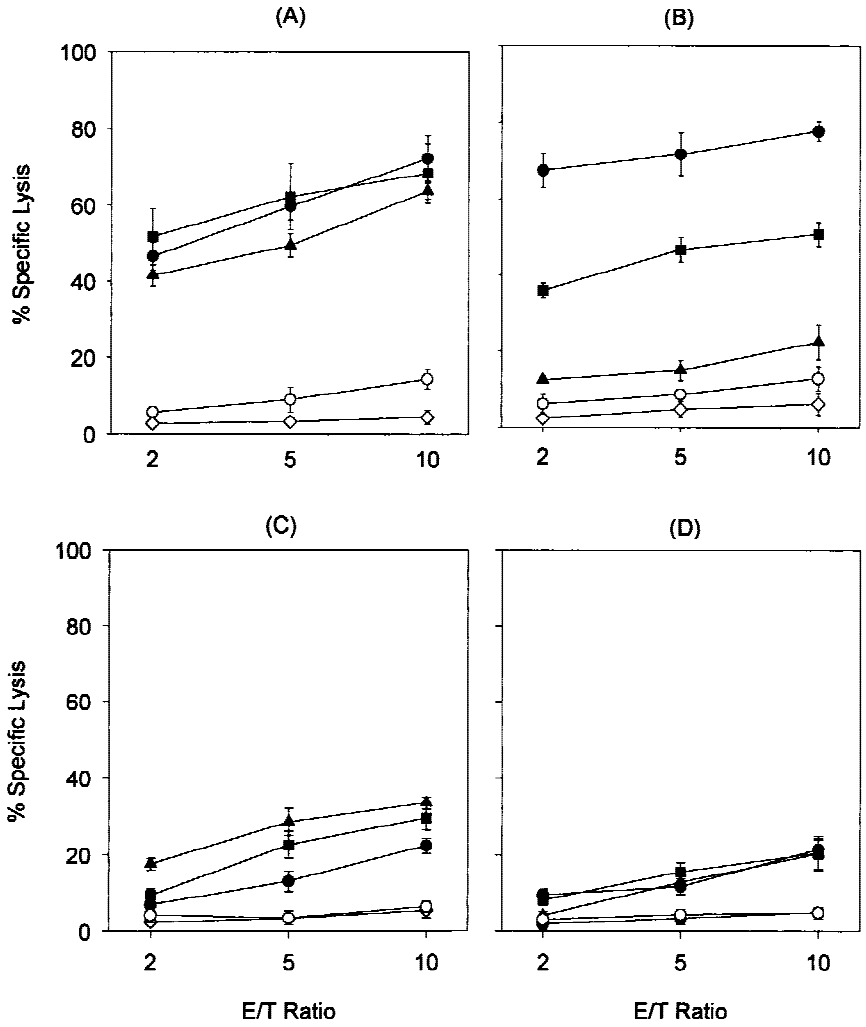

Fig. 1. Cytotoxic activity of bulk-cultured splenocytes. Splenocytes from C57BL/6 mice infected with HTV (10^5 ffu/head) were restimulated in vitro with either NP4 **(A),** NP3 **(B),** NP7 **(C),** or without peptide **(D).** Two mice were used for infection and splenocytes from them were pooled for in vitro stimulation. Irradiated syngeneic splenocytes were used as APC after pulsing with each peptide (10 μg/ml). Effector cells generated after 2 weeks of culture were assayed on ^{51}Cr-labeled EL-4 target cells pulsed with peptide (10μg/ml) NP4 (■), NP3 (●), NP7 (▲), OVA 257–264 (◇), and EL-4 control (○). OVA peptide (SIINFEKL, chicken ovalbumin 257–264) was used as a control of H-2K^b-binding peptides. Data are expressed as mean percentage lysis ± SD (n = 3) and represent 1 example of 3 similar experiments.

NP7) were recognized during a natural HTV infection and 2 peptides (NP3 and NP4) could stimulate anti-HTV CTLs to carry cytotoxic activities over 2 weeks, although cross-reactivity exists, especially in NP4 stimulation.

Induction of CTL Responses by Immunization With HTV NP Peptides

We next examined whether immunization with NP3, NP4, and NP7 peptides would induce peptide-specific CTLs. To evaluate the immunogenicity of peptide, C57BL/6 mice were immunized with peptides in liposome formulation as described in Materials and Methods. Splenocytes were harvested 7 days after the last immunization and restimulated with relevant peptides. Peptide NP3 induced strong NP3-specific CTL responses and nonspecific cytotoxicity was very low, below 10% (Fig. 2B). Peptide NP4 also induced strong NP4-specific CTL activity but nonspecific killing was considerably high, about 20% (Fig. 2A). Peptide NP7, although recognizable by primary anti-HTV CTL responses (Table I), did not elicit CTL responses (Fig. 2C). To further investigate nonimmunogenicity of NP7, peptide NP7Y derived from NP7 by substituting tyrosine at position 5 for proline, was synthesized and tested for peptide-specific CTL inducibility. As shown in Figure 2D, NP7Y-specific CTL activity was induced and also displayed some responses, about 20% at E/T = 5, against NP7 peptide. In H-2K^b-motif, position 5 is known as a primary anchor site, and tyrosine residue is more favorable for that position than proline [Kelly et al., 1993; Feltkamp et al., 1994]. Thus nonimmunogenicity of NP7 seems to be due to the very weak or nonbinding property of the peptide to MHC molecule. The results shown in Figures 1 and 2 demonstrate that NP3 and NP4 are H-2K^b-restricted mouse CTL epitopes in HTV NP and NP3 epitope induces more specific CTL activity than NP4 epitope.

Generation of HTV NP3-Specific CTL Clones

Hitherto, bulk-cultured anti-HTV CTL was used to investigate the recognition of peptides and the immunogenicity of peptides. Those CTL activities might be the sum of the total CTL population, so cross-reactivity was unavoidable. To stringently address the effect of

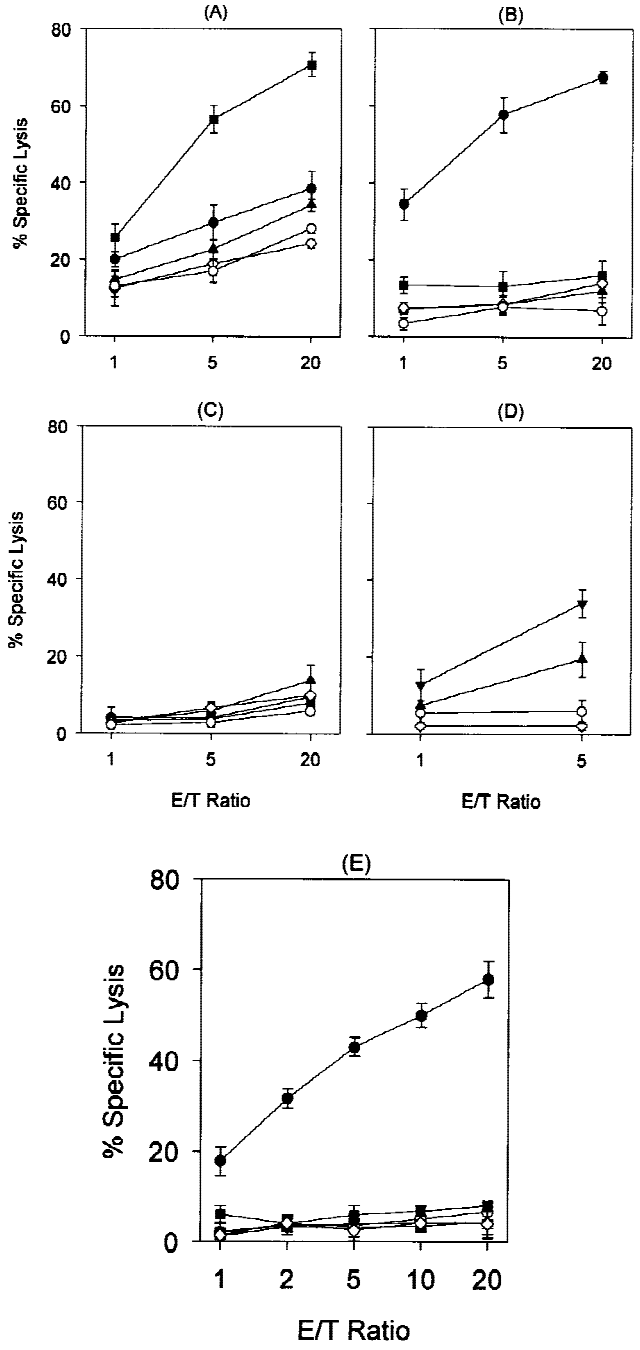

Fig. 2. Induction of peptide-specific CTL responses by immunization with peptide epitopes and cytotoxic activity of NP3-specific CD8+ CTL clone HT-NP3-1-1. C57BL/6 mice were immunized i.p. with 100 μg/0.1 ml/dose of either NP4 **(A),** NP3 **(B),** NP7 **(C),** or NP7Y **(D)** peptide twice at 7-day intervals and splenocytes were restimulated in vitro for 2 weeks as described in Materials and Methods. Three mice were used per immunization group. As targets, EL-4 cells were pulsed with each peptide (1.0 μg/ml). NP7 (SNQEPLKL) was modified by P5 to Y5 substitution. This NP7Y peptide was used to immunize C57BL/6 mice and assessed for CTL induction. Targets were EL-4 pulsed with NP4 (■), NP3 (●), NP7 (▲), OVA 257–264 (◇), NP7Y (▼), and EL-4 negative control (○). **E:** To show the NP3-specific CTL responses of HT-NP3-1-1 clone, EL-4 cells were sensitized with each peptide (0.1 μg/ml), i.e., NP4 (■), NP3 (●), OVA 257–264 (◇), or used as negative control (○). P815 (H-2^d) (▲) and YAC-1 (H-2 K^kD^d) (▼) were also used as target cells to assess the H-2^b restriction and non-NK-like cytotoxicity of this clone. Data are expressed as mean percentage lysis ± SD (n = 3) and represent 1 example of 3 similar experiments. Other clones also showed similar results.

TABLE II. Stability of CTL Clones After Long-Term In Vitro Stimulation

Clone	Immunization[a]	Specific lysis (%) at weeks after cloning[b] 0	3	8
HT-NP4-1-2	NP4-lip. (i.p.) × 2	28.4	12.4	6.8
HT-NP4-1-3	NP4-lip. (i.p.) × 2	39.6	5.0	n.g.[c]
HT-NP4-2-5	NP4-lip. (i.p.) × 3	30.1	7.8	n.g.
HT-NP4-2-7	NP4-lip. (i.p.) × 3	25.8	10.2	5.2
HT-NP4-3-9	HTV	28.7	2.4	n.g.
HT-NP3-1-1	NP3-lip. (i.p.) × 2	50.4	52.7	49.5
HT-NP3-1-4	NP3-lip. (i.p.) × 2	32.0	38.0	36.1
HT-NP3-2-2	NP3-lip. (i.p.) × 3	45.6	42.1	39.5
HT-NP3-2-5	NP3-lip. (i.p.) × 3	39.5	36.5	38.8
HT-NP3-3-1	HTV	44.3	39.6	46.5
HT-NP3-3-2	HTV	33.2	35.0	37.2
HT-NP3-3-3	HTV	37.0	20.3	2.7

[a]C56BL/6 mice were immunized i.p. with HTV (10^5 ffu) or with 100 μg peptide/dose in lipid (lip.) formulation twice or thrice at 7-day intervals. Two mice were used per immunization group. Splenocytes from each mouse were harvested 1 week after the last immunization.
[b]CTL activity was assessed at 3 and 8 weeks from limit-dilution cloning. CTL clones were maintained by in vitro restimulation with irradiated syngeneic splenocytes prepulsed with peptide (NP4 or NP3, 10 μg/ml). EL-4 cells were pulsed with 0.1 μg/ml peptide (NP4 or NP3). E/T ratio was 3. Specific lysis was calculated by subtracting the lysis of control cells (EL-4) from the lysis of target cells.
[c]n.g., not grow. Data are expressed as the average of triplicate assays. Cloning experiments were performed at least twice per immunization group.

epitope peptides on the functional properties of CTLs, a panel of CD8+ CTL lines was generated by 3–4 cycles of restimulation of bulk-cultured CTLs and isolation of CD8+ T lymphocytes followed by limit-dilution cloning as described in Materials and Methods. NP4-reactive CTLs, after multiple restimulations, retained epitope-specific CTL activity at the time of cloning, although the CTL activity was reduced to half of the 2-week cultured CTL activity as shown in Figure 2A and Table II. These CTLs showed very low CTL activity at 3 weeks from cloning and finally lost cytotoxicity and/or did not proliferate any more from 8 weeks. As shown in Table II, NP4-specific CTL clones could not be generated from either peptide-immunized mice or HTV-immunized mice. In addition, subsequent attempts to establish NP4-specific CTL clones resulted in the repeated isolation of cells that lost cytotoxic activity or did not proliferate any more. However, NP3-specific CTL clones were derived from both NP3-immunized splenocytes and HTV-immunized splenocytes. There were some CTL monoclones even in case of NP3 stimulation (Table II), e.g., HT-NP3-3-3, which lost CTL activity and/or did not proliferate from 8 weeks after cloning.

Finally, NP3-specific CTL monoclones were established from 3 independent cloning experiments (Table II). These clones have been stably maintained and retained epitope-specific cytotoxicity over 2 years. One of them, clone HT-NP3-1-1, was highly specific for the HTV NP3 peptide (SVIGFLAL) and exhibited vigorous lysis of target cells (Fig. 2E). The extent of lysis of YAC-1 and P815 (H-2^d) target cells was found to be negligible and similar to that obtained when using negative control EL-4 cells not pulsed with peptide

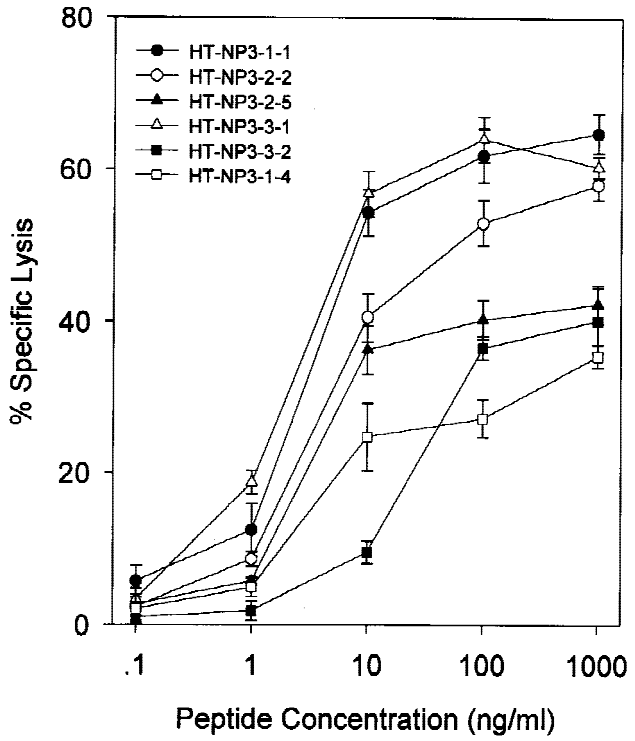

Fig. 3. Sensitivity of CTL activity of NP3-specific CTL clones. The relative ability of the CTL clones to recognize NP3 epitope peptide was assessed in a 4-hour standard ^{51}Cr-release assay. As targets, EL-4 cells were pulsed with NP3 peptide at various concentrations and E/T was 3:1. Data are expressed as mean percentage lysis ± SD (n = 3) and represent 1 example of 3 similar experiments.

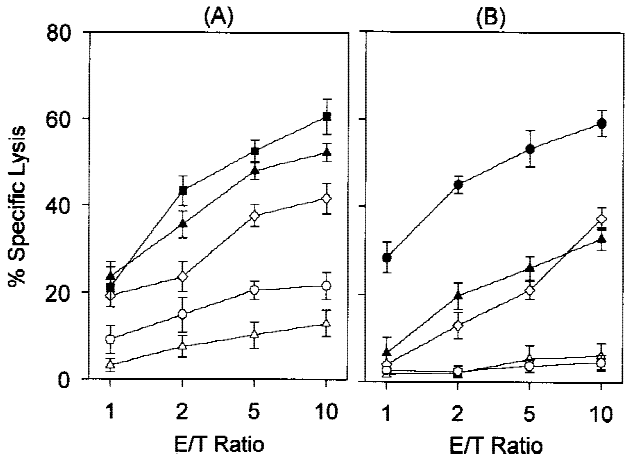

Fig. 4. Recognition of target cells expressing HTV NP endogenously by CTL. As target cells, macrophages infected with HTV (▲) and HTV NP-transfected EL-4 cells (◇) were used as described in Materials and Methods. NP3 (●)- or NP4 (■)-pulsed EL-4 cells, normal macrophages (△), and EL-4 cells (○) were used as negative control. **A:** Bulk-cultured splenocytes from NP4-immunized C57BL/6 mice were re-stimulated in vitro with NP4 peptide for 2 weeks and used as effector cells. Two mice were used per immunization group and splenocytes from them were pooled for further experiments. **B:** NP3-specific CD8+ CTL clone HT-NP3-1-1 was used as effector cells. Data are expressed as mean percentage lysis ± SD (n = 3)and represent 1 example of 3 similar experiments. Other clones also showed similar results.

(Fig. 2E). This result suggests that CTL activities elicited by HT-NP3-1-1 were not due to natural killer (NK) cell activity or H-2^d-restricted responses. Other NP3-specific CTL clones also showed similar results. NP3 peptide titration experiments displayed dose-dependent cytotoxicity of NP3-specific CTL clones. These clones showed comparable recognition of the epitope peptide NP3. CTL activity elicited by these clones seemed to be saturated in the target-sensitizing condition around 10 μg/ml of NP3 peptide at E/T ratio of 3:1 (Fig. 3).

The cell surface phenotype of NP3-specific CTL clones was characterized by FACS analysis (data not shown). Monoclone HT-NP3-1-1 was CD3+, CD4−, CD8+, CD62L−, CD25+, CD122+, NK1.1−, and DX-5−. This result means that HT-NP3-1-1 clone is CD8+ CTL (CD3+, CD4−, CD8+), activated (CD62L−, CD25+, CD122+), and not NK cells (NK1.1−, DX5−). Other clones also showed the same results, but some clones showed NK1.1$^{dim/+}$.

Recognition of Endogenously Expressed Antigens With CTL Responses

It was reported that CTL cell lines generated by in vitro stimulation, especially with high concentrations of epitope peptide, often cannot lyse targets expressing antigens endogenously [Alexander-Miller et al., 1996]. So we assessed HT-NP3-1-1 clone for recognizing targets prepared by infection of macrophages with HTV or by transfection of EL-4 with HTV nucleoprotein as described in Materials and Methods. Bulk-cultured anti-HTV CTLs were used as effector cells after in vitro restimulation with NP4 peptide to examine the endogenous processing and presentation of NP4 peptide. Both NP4-restimulated bulk-cultured CTLs (Fig. 4A) and NP3-specific CTL clones (Fig. 4B) recognized not only HTV-infected targets but also NP protein-transfected targets, although the cytotoxicity was lower than the activity achieved from targets pulsed with relevant epitope peptide. These results demonstrate that NP4-restimulated CTLs and NP3-specific CTL clones could recognize endogenously processed antigens. Furthermore, it is implied that HTV NP is endogenously processed resulting in presentation of epitopes containing NP3 and NP4.

TCR Vβ Typing

Several reports have shown that a limited number of TCR Vβ gene segments are used by CTL clones recognizing the same antigenic determinant [Cose et al., 1995; Lehner et al., 1995]. We set out to determine whether this was also true for the HTV NP3-specific CTL clones. CTL clones were stained separately with 14 different murine Vβ-specific antibodies and analyzed with FACSCalibur flow cytometry as shown in Figure 5. This analysis revealed that the NP3-specific clone HT-NP3-1-1 did not bind other anti-Vβ antibodies (Fig. 5A-2,3) than anti-Vβ6 antibody (Fig. 5A-1). HT-NP3-1-4, HT-NP3-3-1, and HT-NP3-2-2 clones also showed Vβ6 expression (Fig. 5B–D). All of the other clones preferentially expressed the same TCR Vβ element, Vβ6.

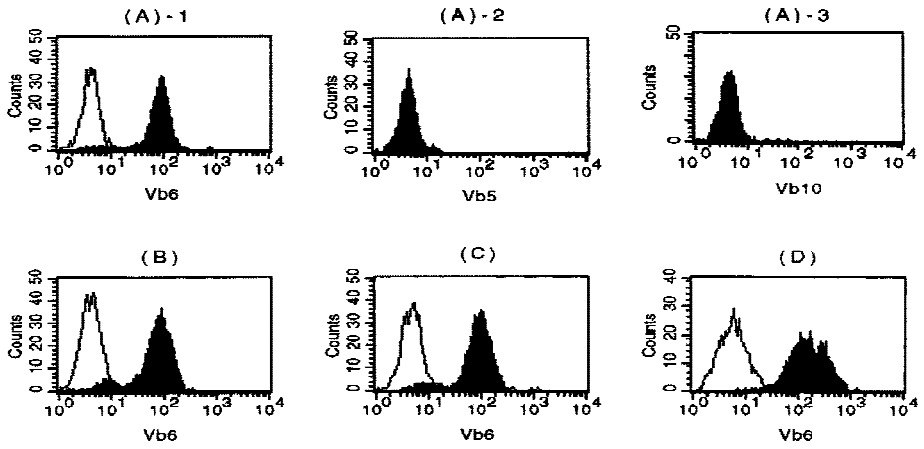

Fig. 5. Typing of TCR Vβ on NP3-specific CTL clones HT-NP3-1-1 **(A-1),** HT-NP3-1-4 **(B),** HT-NP3-3-1 **(C),** and HT-NP3-2-2 **(D).** Cells were stained separately with 14 different anti-TCR Vβ antibodies labeled with PE or FITC. Cells only and stained cells are shown as open and filled histograms, respectively. Data from staining with anti-Vβ6 are shown. Results from other anti-Vβ antibodies showed a staining pattern identical to that of Vβ5 **(A-2)** or Vβ10 **(A-3),** which is shown as an example of negative staining. All other clones were also tested and shown to express preferentially Vβ6.

DISCUSSION

CTLs are an important host defense mechanism against many viral infections especially in clearance of virus-infected cells. In mice, adoptive transfer experiments have demonstrated that CTLs mediate protective immunity against LCMV, herpes simplex virus, and influenza virus [Zinkernagel and Doherty, 1979; Sethi et al., 1983]. In humans, evidence for a protective role of CTL against viral infection arises from the observation of a positive correlation between virus-specific CTL activity and recovery from infection with cytomegalovirus or influenza virus [Quinnan et al., 1982; McMichael et al., 1983].

There are many differences between the mouse infection with HTV and the human disease, HFRS, caused by HTV. HTV infection in adult mice results in transient viral replication with no symptom followed by the elimination of virus. But HFRS is a severe, life-threatening illness characterized by fever, hemorrhage, and renal failure. Although neutralizing antibodies are thought to protect mice and humans from viral infection, CTL responses after HTV infection show several differences between mice and humans. The role of CTL responses for protection and/or recovery from HFRS in humans is not known. Recent studies of HTV and related virus, SNV, suggest that CTL responses play a role in the pathology of HFRS even if the pathogenesis is not understood [Ennis et al., 1997; Van Epps et al., 1999].

In the murine model studied in many reports, CTLs are considered to be important for in vivo clearance of HTV. Adoptive transfer of immune T cells protected suckling mice from death following infection with HTV [Nakamura et al., 1985a; Asada et al., 1987]. Others reported that T-cell-mediated immunity plays an important role in resistance of mice to HTV infection [Nakamura et al., 1985a; Asada et al., 1988; Yoshimatsu et al., 1993]. It was also found by transferring T-cell subsets into HTV-infected nude mice that T cells expressing CD4– CD8+ markers on their surface were especially important for elimination of infectious viruses in vivo [Asada et al., 1987]. Although both virulent and nonvirulent HTV clones elicited similar titers of antibodies in mice, CTL activity was detected only in mice infected with nonvirulent clones[Tamura et al., 1989; Isegawa et al., 1994]. It is known that the neutralizing antibodies eliminate extracellular viruses, whereas CTL responses destroy the virus-infected cells from which viruses spread. In this case also, CTL responses elicited by nonvirulent HTV clones might destroy the virus-infected cells and limit the systemic spreading of viruses. However, virulent clones did not induce CTL responses against HTV, therefore viruses could spread from the HTV-infected cells resulting in systemic infection. These findings indicate the importance of further studies on murine CTL responses to HTV.

In this study, we have identified 3 H-2K^b-restricted CTL epitopes in HTV NP: NP3 (NP221–228, SVIGFLAL), NP4 (NP328–335, LGAFFSIL), and NP7 (NP422–429, SNQEPLKL). Of course, the binding algorithm used in this study cannot predict all of the T-cell epitopes within HTV NP. In consideration of this probability and to obtain qualitative information about the overall CTL responses to HTV infection, we also used HTV-infected PEC and NP-transfected EL-4 cells as target cells. These endogenous target cells were recognized by primary anti-HTV CTLs, and CTL activities against them were somewhat higher than those against peptide-pulsed targets. Although these results could not be compared directly with those of peptide-pulsed targets, it cannot be excluded that CTL responses elicited by HTV infection also contain cytotoxicities directed against epitopes other than NP3, NP4, and NP7.

This experiment was performed in a direct ex vivo cytotoxicity assay, so CTLs recognizing less dominant epitopes might not have been stimulated thoroughly enough to expand [Lipford et al., 1995; Van der Most et al., 1998]. To examine this possibility, we measured secondary CTL responses after in vitro stimulation. After in vitro secondary stimulation with NP3 or NP4 peptide, CTL activity against the relevant peptide was increased and cross-reactivity was decreased. This implies that specific CTL precursors can be amplified and

nonspecific CTLs will not be stimulated by this experimental approach [Murali-Krishna et al., 1994].

The induction of a CTL response requires that antigens gain access to the class I presentation pathway, which occurs in the cytosolic compartment of the APC [Yewdell et al., 1988]. A number of vaccine delivery systems including ISCOM, DNA, lipopeptide, and viral vectors have been developed to induce CTL responses [Catarina et al., 1996; Lee et al., 1998]. It is known that liposome can deliver encapsulated substances, including drugs and antigens, into the cytosol [Zhou et al., 1992]. In many studies in which successful CTL responses were induced, liposome-antigen formulations were used as antigen delivery systems [Collins et al., 1992; Zhou et al., 1992; Alving and Wassef, 1994]. We also used liposome formulations to incorporate peptide antigen and to deliver into the class I pathway.

NP7Y induced CTLs, which lysed NP7-sensitized targets also. This result probably reflects that CTLs elicited with substituted epitope (NP7Y) can recognize the wild-type peptide (NP7). Therefore, it is possible that the immunogenicity of subdominant epitopes can be enhanced by increasing the binding affinity to MHC molecule as reported by others [Bakker et al., 1997; Tourdot et al., 1997]. The cross-reactivity in the primary HTV CTLs was greatly reduced in CTLs induced by NP3 peptide immunization. This result shows that HTV immunization induces diverse CTL responses, but more specific CTL responses are elicited by immunization with the epitope peptide. However, CTLs induced by NP4 peptide immunization still showed significant cross-reactivity (20–30% lysis) to other epitope peptides. It was reported that the CTL population specific for the same epitope peptide displayed cross-reactive cytotoxicity to other epitopes [Kessler et al., 1997]. This can confer some advantages to host immune responses against viral infection. In our experiments, NP4 evoked more diverse and vigorous CTL responses than NP3.

CTL clones specific for an epitope peptide are useful for the investigation of the roles of the epitope peptide and CTL responses in hosts infected with virus. Positions 5 and 8 are known as anchor sites for H-2 K^b molecule and positions 4, 6, and 7 are TCR-binding sites [Feltkamp et al., 1994; Udaka et al., 1995]. To examine this property of the NP3 peptide, we tested CTL clone HT-NP3-1-1 for the recognition of peptides with individual alanine substitution at each of the 8 residues of NP3 sequence (SVIGFLAL) except for position 7, which was replaced with glycine. CTL responses to these peptides by HT-NP3-1-1 clone were compared with the specific CTL activity against NP3-sensitized targets (data not shown). Substitution of alanine for phenylalanine at the fifth residue or leucine at the eighth position drastically inhibited the recognition by CTLs. Substitution of alanine for serine at the first residue did not impair recognition, and substitution at the second and third positions allowed considerable CTL activities. However, substitution at the fourth, sixth, and seventh residues greatly inhibited peptide recognition by clone HT-NP3-1-1. These results were expected considering that positions 4, 6, and 7 are known as possible TCR-contact residues in H-2K^b-restricted recognition [Udaka et al., 1995; Spaulding et al., 1999]. Therefore, HT-NP3-1-1 shows typical characteristics of H-2K^b-restricted CD8+ CTL clones. Other clones also showed similar results.

It is reported that CTL clones specific for the same epitope use an identical TCR Vβ type. For example, Vβ10 is preferentially used by H-2D^b-restricted CD8+ CTLs specific for LCMV GP2 275–289 [Yanagi et al., 1990] and Vβ5 by H-2K^b-restricted CD8+ CTL clones for OVA 257–264 [Dillon et al., 1994]. All of 6 NP3-specific CTL clones showed the restricted usage of TCR Vβ6. It is difficult to conclude that CTLs using Vβ6 are predominant in the immune response to HTV inoculation, because it cannot be excluded that T cells expressing other Vβ segments also could recognize the peptide epitope NP3. Therefore, further analysis is required to ascertain whether a preferential expansion of CD8+ T cells using Vβ6 accompanies HTV infection in vivo.

A possible concern regarding CTL epitopes, identified by peptide sensitization of targets followed by recognition with CTL responses, is whether these peptides are processed and presented in endogenous condition. Asada's group [1988] used macrophages, after infection with HTV, as target cells for CTL activity assay of splenocytes from HTV-immunized mice. The infected macrophage is thought to be responsible for the spread of hantaviral infection in rodents and humans. This may be caused by the enhanced attachment of the virus-antibody complex to the Fc receptor on the macrophage [Yao et al., 1991]. Recently it was reported that the cellular entry of HFRS-associated hantaviruses is facilitated by β3 integrins, which are present on the surface of macrophages [Gavrilovskaya et al., 1999]. Therefore, it is worthwhile to investigate whether NP3-specific CTL clones could lyse macrophages infected with HTV. OVA-transfected EL-4 cells also were used as endogenous target cells for the cytotoxicity assay of OVA 257–264 peptide-specific CTL clones [Ke et al., 1998]. Based on the results from Figure 4, NP3 and NP4 peptides are thought to be endogenously presentable epitopes. This implies that these epitopes could be utilized for the investigation of the importance of murine CTL epitopes and/or their CTL responses in HTV infection.

It is still controversial whether neutralizing antibodies alone are sufficient or whether cytotoxic T-cell responses are required for efficient protection against HTV infection. Although the murine model and human disease are different in many respects after HTV infection, studies regarding CTL epitopes and CTL responses in mice after HTV infection could give insights into how CTL responses might work in natural infection. In conclusion, we have identified murine H-2K^b-restricted CD8+ CTL epitopes and established murine CTL clones specific for an epitope, NP3, in HTV NP. These epitopes and NP3-specific CTL clones might be useful for both the design of HTV vaccine strategies

and the investigation of the roles of CTLs as protective immune responses in HTV infection.

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